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Molecular Evaluation of *FLC* Homologs and Coordinating Regulators on the Flowering Responses to Chilling Temperature in Cabbage (*Brassica oleraceae* var. *capitata*) Genotypes

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Abstract: Cabbage (*B. oleracea* var. *capitata*) contains three *FLC* homolog genes (*BoFLC1*, *BoFLC2*, and *BoFLC3*) and one pseudogene (*BoFLC5)* that responds to low temperatures necessary for flowering. We isolated three *BoFLC*-encoding genes from early-, mid-, and late-flowering cabbage plants. Leaf samples were collected for RNA extraction and expression analysis. Gene structure analysis and phylogenetic comparison were performed for intra- or interspecific relationships of the *BoFLC* homologs. Gene expression related to flowering regulators (*BoGI*, *BoCOOLAIR*, and *BoVIN3*) was analyzed. While *BoFLC* genes commonly have seven exons and six introns of 3,361–4.384 bp, variations in insertion or deletion were evident between the early- and late-flowering genotypes. Repressed expression of *BoFLC 1*, *2*, and *3* genes under chilling temperatures appeared to occur from 8 weeks after vernalization. Higher expression levels of *GI*, *COOLAIR*, and *VIN3* were initiated 7 weeks after chilling (WAT) treatment in the early flowering genotype. The vernalization trigger repressed the expression of *BoFLC* homologs. This study provides molecular insights into *BoFLC* homologs between early- and late-flowering cabbage genotypes, in which structural variations in *BoFLC1* appeared to be important as a binding motif to flowering regulating factors such as *BoGI*, *BoCOOLAIR*, and *BoVIN3* for which motif analysis is further implemented.

Keywords: *BoFLC* homologs; cabbage flowering; flowering regulator; structural variation; vernalization

1. Introduction

In plants, the regulation of flowering time is one of the most important events that significantly affect the synchronization of reproduction under favorable environmental conditions. Floral transition, such as the vegetative-to-reproductive phase, is a vital developmental process that governs the timing of reproduction. Environmental (photoperiod and temperature) and endogenous factors (autonomous, gibberellins [GA], and aging), functionally already characterized in *Arabidopsis*, regulate this floral transition [1–4]. Cold temperature-induced floral transitions such as vernalization are widely present in long-day plants in biennial or winter forms. This vernalization pathway comprises many genes that act as transcriptional and epigenetic silencing mechanisms to form a complex regulatory network that controls flowering time.

Brassicaceae are some of the most economically important crop species in horticulture and are cultivated mainly for their edible seeds, stems, leaves, and flowers. In spring, crops in this family require prolonged vernalization to cause premature flowering, which results in bolting and

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impairing yield [5]. Therefore, breeding late-flowering cabbages that are resistant to cold temperatures and bolting is of considerable interest to breeders and researchers[6,7]. Important vernalization components, along with flowering-related genes (*FLC*, *FRI,* and *VIN3*), are evolutionarily conserved between the spring and winter forms of *Arabidopsis* and *Brassica* crops[8–12]. Natural variation in flowering time between spring and winter forms is mostly associated with allelic variations in paralogs of *FLC* locus, which has already been characterized in many *Brassica* species, including *B. oleracea*[13–15], *B. rapa*[16,17], *B. napus* [18,19].

The reference genome of *B. oleracea* contains four copies of *BoFLC1/2/3* and a pseudogene of *BoFLC5* orthologs[10,20]. In earlier studies, only *BoFLC2* was the main functional gene responsible for floral transitions[10,14]. Supporting this, mutations in *BoFLC2* cause a loss-of-function in annual cauliflower varieties[13]. A recent transformation study showed that three *BoFLC*s delayed flowering in *Arabidopsis* and suggested that these paralogs were functionally conserved as floral repressors in cabbage[21]. However, the investigated mRNA expression characteristics revealed that *BoFLC1* declined less markedly than the highly repressed genes *BoFLC2* or *3* under cold treatment[21]. In addition, an insertion mutation in intron 2 of *BoFLC1* and its rapid downregulation under vernalization was strongly associated with the vernalization-dependent phenotype of an early flowering cabbage line. Allelic diversity and variations in *FRI* have also been closely linked to flowering or heading date variations in *B. oleracea* [22,23] and *B. napus* [24].

To date, variations in flowering time and vernalization sensitivity among the contrasting flowering time (early, mid-, and late) phenotypes of cabbage cultivars have been inadequately investigated. In this study, we characterized three *BoFLC* functional members, *BoFLC1*, *BoFLC2*/*4*, and *BoFLC3*, in the early-, mid-, and late-flowering cabbage cultivars. We found that allelic variations at the *BoFLC* locus could alter vernalization sensitivity between cabbage cultivars. This also suggests that these allelic nucleotide variations may influence the epigenetic silencing of *FLC*, as noted in *Arabidopsis.* Therefore, we propose that the identification of cis polymorphisms in *BoFLC1*, *BoFLC2,* and *BoFLC3* provides beneficial information for breeding *Brassica* crops resistant to cold and late-flowering lines.

Arabidopsis contains a key gene for repressing floral transition called *Flowering Locus C (FLC)*, which encodes a MADS-box transcription factor[25]. During vernalization, floral integrators, including *Flowering Locus T (FT)*[25], and transcription factors of MADS-box encoding proteins, such as *Suppressor of Overexpression of Constans1 (SOC1)* [26]and *Leafy (LFY)* [27,28] are activated, consequently promoting flowering by reducing the gene product of *FLC*. *Gigantea (GI)*, a circadian clock-associated protein, is involved in various physiological processes, including the regulation of flowering time[29]. *GI* positively regulates *Constans (CO)* and *FT*, suggesting a role in floral transitions[30,31].

Extended vernalization induces genetic and epigenetic regulatory mechanisms that control the transcription of *FLC* by stabilizing histone proteins. Under non-vernalization conditions, *FLC* expression is increased by an activator called *FRIGIDA (FRI),* which recruits chromatin modification factors to the *FLC* chromatin, thereby repressing flowering time. Natural antisense transcripts (NATs) play pivotal roles in the epigenetic silencing of *FLC* [32]. Cold temperature induces the transcription of noncoding sense and antisense transcripts of *COLDAIR* and *COOLAIR* from the first intronic and multi-exonic or promoter adjacent 3′ regions in the *FLC* locus, respectively[33,34]. *COLDAIR* aids in the addition of repressive factors to *FLC* chromatin through interaction with *VERNALIZATION INSENSITIVE 3* (*VIN3*) by recruiting the PRC2-like complex, a group of polycomb proteins, to the histones of *FLC*[34]. Independently from this, but before the decrease of *FLC* expression by the PRC2-like complex, antisense transcripts of *COOLAIR* are produced, repressing the sense transcripts of *FLC* [33].

2. Results

2.1. Molecular characterization of BoFLC-encoding genes from different cabbage bolting and flowering lines (variation in gene structures)

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Experiments were conducted on cabbages with different flowering periods (CAB1: early-flowering at 140–150 days, CAB3: mid-flowering at 160–170 days, and CAB5: late-flowering at ≥ 190 days). Genomic DNA sequences ranging from approximately 3361 bp to 4384 bp for *BoFLC1*, *BoFLC2*, and *BoFLC3*, and CDS and untranslated regions of 594 bp were obtained (Figure 1). In the *BoFLC1* gene sequence, the difference between CAB1 and CAB5 was in intron 1. Insertions of 23 and 335 bp were in intron 1 of CAB5 (Figure 1A). *BoFLC*2 also had a deletion in intron 1; however, there was no difference in *BoFLC3* (Figure 1B-C). The reference and *BoFLC* gene sequences were compared (Figure S1). In the *BoFLC1* gene sequence, 687 and 67 bp insertions were in introns 1 and 2, respectively, in early flowering type cabbage (CAB1), mid-flowering type cabbage (CAB3), and late-flowering type cabbage (CAB5), compared to the previously known AM231517.1 sequence. Unlike CAB1, CAB3,5 had 23, 613, and 5 bp deletions and 3, 9, 9, and 13 bp insertions in intron 1. CAB1 had 70, 40, 13, 29, and 78 bp deletions in introns 5 and 6, and CAB2 had 70, 70, 40, 5, 13, 29, and 13 bp deletions in introns 5 and 6. In CAB3, deletions of 70, 70, 40, 5, 13, 29, and 78 bp were observed in introns 5 and 6 (Figure S1A). In the *BoFLC2* gene sequence, similar to the *BoFLC1* gene, CAB3 and 5 had a 215 bp deletion in intron 1. In intron 6, CAB2 had 9, 2, and 1 bp insertions, 4, 2 bp deletions, and CAB3 had 9 bp insertions, 4, 2 bp deletions. CAB1 was 100% consistent with the previously known AY306124.1 sequence (Figure S1B). Finally, *BoFLC3*, CAB1, CAB3, and CAB5 all had 13 and 21 bp insertions and 2, 2, and 11 bp deletions in intron 1; specifically, it was confirmed that 15 bp insertions only existed in intron 5 in CAB3 (Figure S1C). The percent identity matrix was obtained using CLUSTAL multiple sequence alignment with ClustalOmega (http://www.ebi.ac.uk/Tools/msa/muscle/, version 3.8). *BoFLC1*, *BoFLC2*, and *BoFLC3* were based on the sequences of *Brassica* species (Table S1). SNPs and Indels of the *FLC* were abundant in cabbage species. In genomic DNA, a significant In/Del in Introns was found in all *FLC1*, *FLC2*, and *FLC3* genes (Figure S1), but in the CDS, it was confirmed that the similarity between each gene was high. In addition, the CDS of other *Brassica* species were highly similar (Table S3).

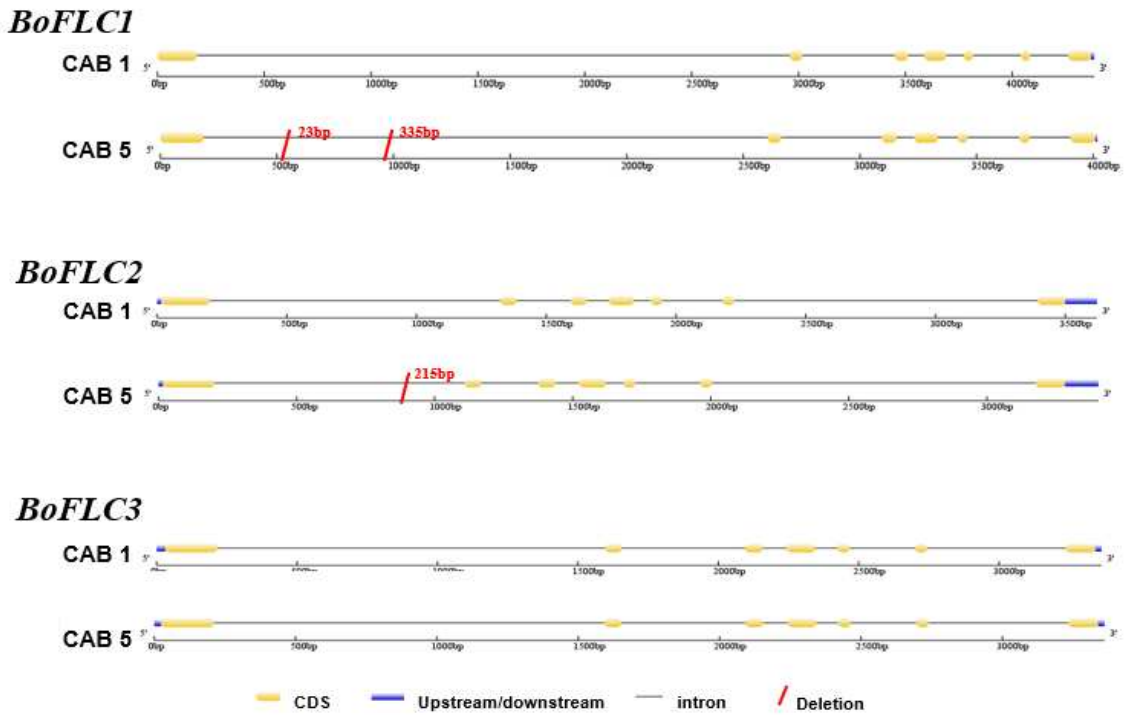

Figure 1. Differences in gene structure of three homologs of *BoFLC1*, *BoFLC2*, and *BoFLC3* between early- (CAB 1) and late- (CAB 5) flowering cabbages. The light-colored regions of the homologs indicate coding sequences and introns, respectively, with upstream and downstream regions in blue boxes. The deleted sequences of *BoFLC1* and *BoFLC2* (CAB 5) were noticed only in the late-flowering genotype (CAB 5).

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2.2. Phylogenetic relationships among B. oleraceae and other Brassica species

We investigated the relationship between the amino acid sequences (*BoFLC1* (CAJ77613), *BoFLC2* (AAQ76275), and *BoFLC3* (CAJ77614), *BrFLC1* (DQ866874.1), *BrFLC2* (DQ866875.1), *BrFLC3* (ABI30001.1), *BnFLC1* (AAK70215.1), *BnFLC2* (AAK70216.1), *BnFLC3* (AAK70217.1), *BniFLC* (AIE38010.1), *BcFLC* (QCE32013.1), *BjFLC* (AHY82602.1)) using the neighbor-joining tree using protein levels (Figure 2). *FLC* homologs were evidently distinguishable from *B. oleraceae* (*Bo*), *B. rapa* (*Br*), *B. napus* (*Bn*), and *B. nigra*. *BoFLC1*, *BoFLC2*, and *BoFLC3* proteins of CAB1 were closely related to the known cabbage proteins. CAB3 and CAB5 were highly correlated with *FLC* homology, indicating that most of the correlations between the proteins of the *FLC* homologs of *B. oleraceae* were close. However, it can be assumed that the complex intron In/Del caused a difference in flowering time.

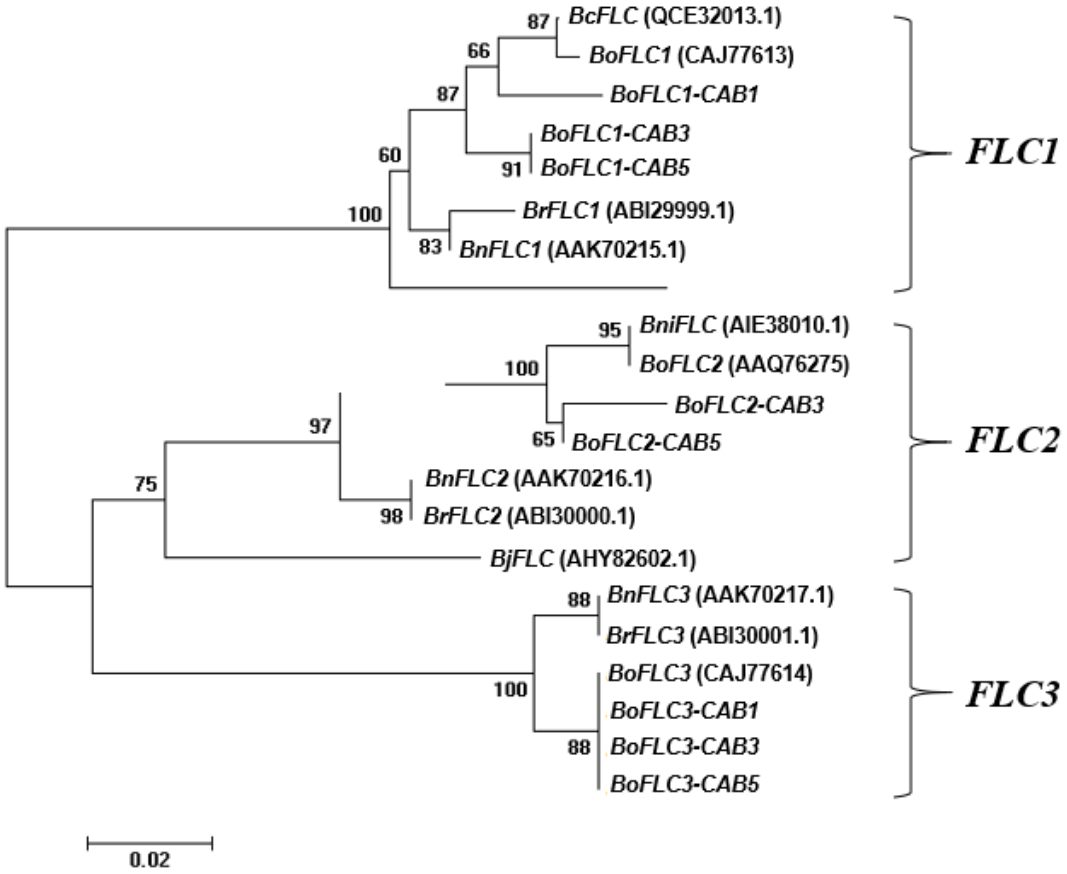

Figure 2. Neighbor-joining tree showing phylogenetic relationships based on the decoded *BoFLC* amino acid sequences among early- (CAB 1), mid- (CAB 3), and late- (CAB 5) flowering cabbages. The GenBank accession number is shown in a parenthesis, and sequences were compared with three cabbage genotypes used in our study. Bootstrap values > 50% are shown above the branches. *Bc, Brassica carinata; Bj, B. juncea; Bn, B. napus; Bni, B. nigra; Bo, B. oleracea; Br, Brassica rapa.*

2.3. Transcriptional expression patterns of BoFLC genes by vernalization

We performed transcript expression analysis using qRT-PCR to determine the effect of cold treatment on the *BoFLC* genes (Figure 3). Mid-flowering (CAB3) and late-flowering (CAB5) cabbages were sampled by low-temperature treatment from 0 to 11 weeks, and RNA was extracted and analyzed for expression from 5 to 11 weeks. Graphs were drawn with the CAB3 treatment, CAB5 control, and CAB5 treatment values as relative values based on the CAB3 control value. First, the controls were maintained without significant differences between the FLC homologs. In addition, the basic expression level in CAB5 was approximately two times higher than CAB3 in the control grown

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in a greenhouse. *BoFLC1* plays a role in inhibiting flowering, and the late-flowering type undergoes significant low-temperature sensitization only after exposure to low temperatures for approximately 7 weeks[15]. Our experimental results confirmed that *BoFLC1* expression decreased rapidly in CAB5 cells at low temperatures for approximately 8 weeks (Figure 3A). At low temperatures, CAB3 decreased *BoFLC1* expression at 8 weeks. However, the expression did not continue to decrease, and *BoFLC1* expression was maintained for 9 weeks in both cabbages under the low-temperature treatment. In the case of the *FLC* homologous gene *BoFLC2*, the expression of both CAB3 and 5 cabbages began to decrease after approximately 7 weeks of cold treatment, and the expression values were maintained until 9 weeks. *BoFLC3* was similar to *BoFLC1* in both CAB3 and the five cabbage varieties.

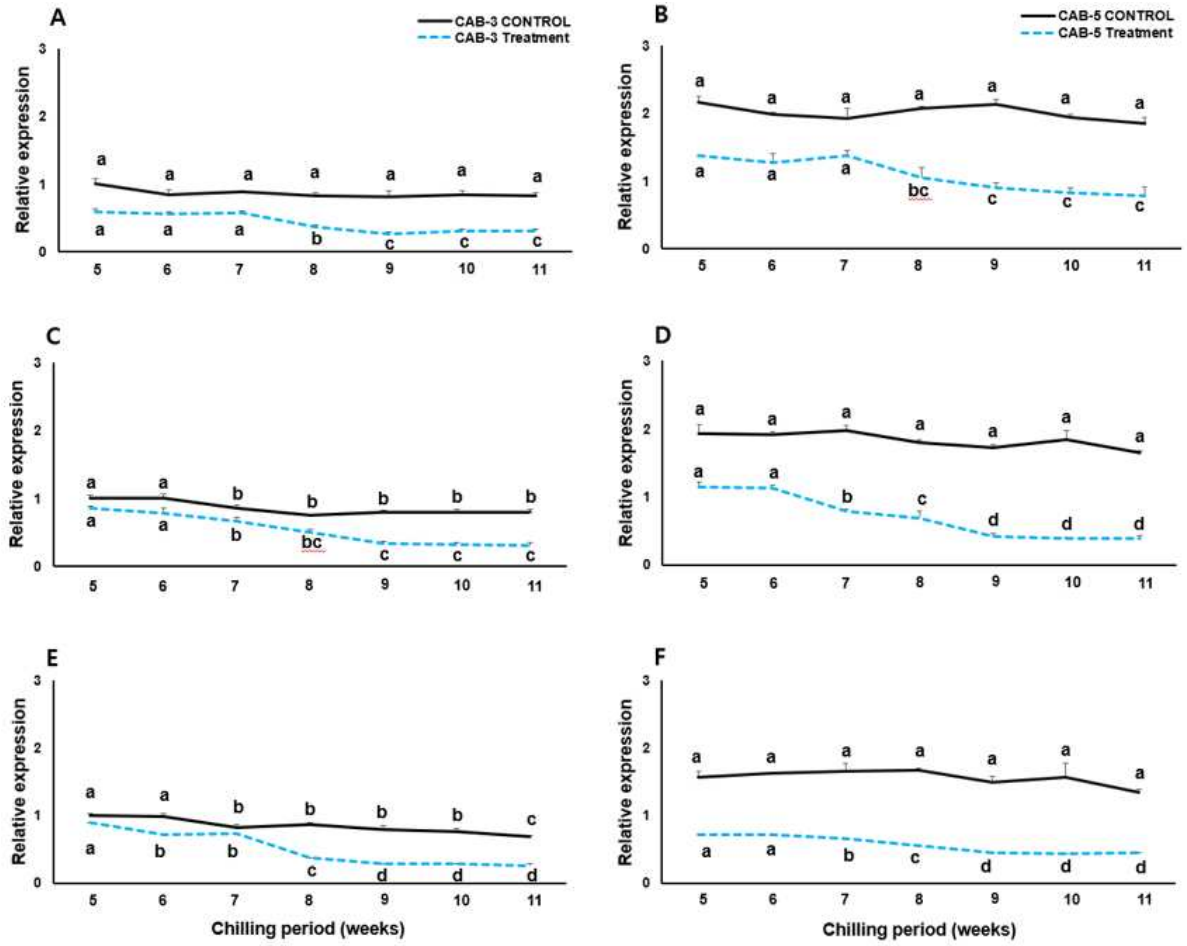

Figure 3. mRNA expression patterns of three *BoFLC* homologs using cDNA from chilling (dotted line) and no-chilling (solid line) treatment between mid- (CAB 3) and late- (CAB 5) flowering cabbages. Relative expressions of *BoFLC1* (top panels A and B), *BoFLC2* (middle panels C and D), and *BoFLC3* (bottom panels E and F) are shown for the mid-flowering CAB 3 (left panels A, C, and E, respectively) and late-flowering CAB 5 (right panels B, D, and F, respectively). Different letters indicate statistically significant differences in gene expression across chilling periods according to Duncan's multiple range test at $p \leq 0.05$.

2.4. Vernalization response of BoGI, BoCOOLAIR, and BoVIN3 in cabbage

In addition to *BoFLC*, qRT-PCR analyses of *BoGI*, *BoCOOLAIR*, and *BoVIN3* were also performed to investigate the expression of flowering-related genes. *GIGANTEA (GI)*, which is related to the circadian clock and the control of flowering time, had a faster time to peak than the control. *GI* is controlled by temperature and light[37]. In the treatments we tested, the peak appeared faster for approximately 2 weeks compared to the control (Figure 4A). The expression of CAB3, which peaked at 9 weeks in the control group, was highest at 7 weeks after cold treatment. For CAB5, only the

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timing of expression was delayed, but the trend was the same; however, a difference existed in the amount of expression. In *Arabidopsis thaliana*, *COOLAIR* plays a role in regulating *AtFLC* expression as a long non-coding RNA[33,34]. We performed qRT-PCR to determine the trend of *COOLAIR* expression in cabbage plants (Figure 4B). Both CAB3 and CAB5, which continued the low-temperature treatment, showed the highest expression levels at 7 weeks. This coincided with the time when the expression of the *BoFLC* gene began to decrease (Figure 4). In CAB3, it increased rapidly at week 7 and then rapidly decreased again. In contrast, for CAB5, it increased more slowly and then gradually decreased. Among the vernalization pathway genes related to flowering, *VIN3* inhibits the transcription of *FLC* by methylation through a series of processes that deacetylate specific regions of *FLC* chromatin, along with histone deacetylases (HDAC) and DNA recognition proteins in cold *Arabidopsis thaliana*[4]. The highest expression was observed at 7 weeks in the CAB3 low-temperature treatment and at 9 weeks in the late-flowering type low-temperature treatment. In CAB3, expression decreased after 8 weeks but was maintained after 9 weeks, whereas in CAB5, expression decreased continuously after 9 weeks.

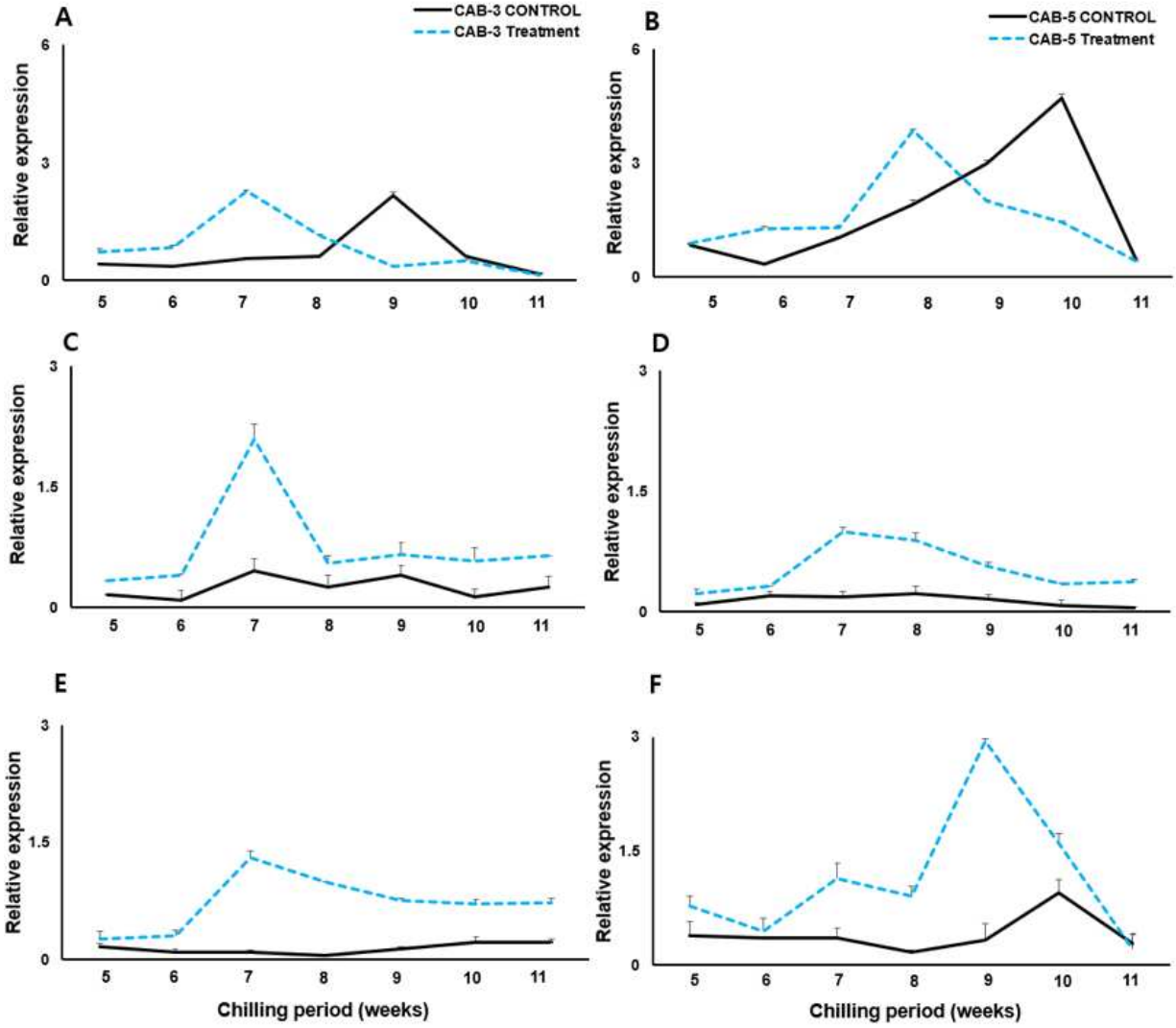

Figure 4. mRNA expression patterns of the flowering-time regulator genes *GIGANTEA* (*GI,* top panel A and B), *BoCOOLAIR* (middle panel C and D), *BoVIN3* (bottom panel E and F) between mid- (CAB 3) and late- (CAB 5) flowering cabbage lines under chilling (dotted line) and no-chilling treatment. The relative expressions of *GI*, *BoCOOLAIR*, and *BoVIN3* were compared for the mid-flowering CAB-3 line (left A, C, E) and late-flowering CAB-5 (right B, D, F) line. The bars indicate standard errors of the means for each chilling week.

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3. Discussion

Various environmental and genetic factors affect flowering. Low temperatures are a major factor that essentially promotes flowering. Among the various genes that regulate flowering at such low temperatures, *Arabidopsis* contains a key gene that inhibits flower metastasis called *FLOWERING LOCUS C* (*FLC*), which encodes a MADS-box transcription factor[25]. Differences in *FLC* homolog genes were investigated in cabbage plants at different flowering times. *BoFLC1*, *BoFLC2*, and *BoFLC3* were cloned using genomic DNA and compared to known genes. The sequences of the *BoFLC1* gene (Bo9g173400) and CAB1 were similar, but those of CAB3 and CAB5 were different. *BoFLC1* sequences were also similar in CAB3 and CAB5, but the difference between the two groups was an In/Del in intron 1. There were three deletions and four insertions in CAB3 and CAB5, respectively. This indicates that flowering may be delayed because the antisense function of *COOLAIR* and *COLDAIR* [34] by binding to *FLC* is lost, and they fail to inhibit *FLC*. Notably, In/Del occurred in intron1 of CAB3 (*BoFLC2* and *3*) and CAB5 (*BoFLC2* and *3*). Similarly, the possibility of the loss of *COOLAIR* and *COLDAIR* binding sites, the antisense of *FLC*, can be estimated. Variations in the insertion/deletion (In/Del) ratio in *FLC* introns, including other genes, may affect flowering. To compare the expression levels of FLC, experiments were conducted in a vernalized environment. To determine whether there were differences in the expression of *FLC* homologous genes, RNA was extracted. It was confirmed that the *BoFLC1* gene is sensitive to low temperatures after exposure to low temperatures for approximately 7 weeks in the late flowering type[15]. Similarly, the expression of *BoFLC1* began to decrease after 7 weeks in cold-treated CAB3 and 5 (Figure 3A). Expression values remained constant from weeks 9 to 11. In contrast, the expression of *BoFLC1~3* in the control group remained constant in both CAB3 and 5 from weeks 5 to 11. The pattern of decreased expression after low-temperature treatment was similar. In the case of *BoFLC2*, the expression levels of CAB3 and 5 treated at low temperatures started to decrease after 7 weeks, and the expression remained constant from 9 to 11 weeks. *BoFLC3* showed a trend similar to that of these two genes. Despite the distinct phenotype of the difference in flowering time between CAB3 and CAB5, the expression trends of *BoFLC1*, *BoFLC2*, and *BoFLC3* between the two cabbages were similar during low-temperature treatment. This may be because the CDS sequences of the *BoFLC1~3* genes between the two cabbages were similar, as were the mutations in the introns. In particular, because the expression values of the *BoFLC1~3* genes were lowered in response to low temperatures, this suggests that the three genes may be related to the traits controlling flowering time. If a difference ensued between the two cabbages, the expression of the gene of *BoFLC1~3* was approximately 1.5 to 2 times higher in CAB5 than in CAB3. This is considered as the amount of expression of the *FLC* homologous gene required to inhibit flowering, which varies among species. Furthermore, the expression values of the CAB5 cold-treated sample in *BoFLC1* and the CAB3 control sample in week 11 were similar. This means that CAB5 needs to be cold-treated for a longer period. To confirm the *FLC* homologous similarity between *Brassica oleracea* species, the CDS of three homologous genes (*BoFLC1*, *BoFLC2*, and *BoFLC3*) of cabbage, kohlrabi, and broccoli with different flowering types were compared. When compared based on the *BoFLC1* sequence of CAB1, the similarity was approximately ≥93% (Table S3). When comparing the sequences of different species based on CAB1, the *BoFLC2* gene was also approximately 95% similar, and the *BoFLC3* gene was approximately ≥94%. We compared the expression of *BoGI*, *COOLAIR*, and *BoVIN3* to determine the relationship between expression differences and *BoFLC* according to the vernalization of genes related to flowering (Figure 4). This is because the expression patterns of the *BoFLC* homologous genes are similar. A difference existed in *BoGI* between the cold-treated and control groups. In CAB3, the peak appeared approximately two weeks earlier in the cold-treated group than in the control group. The same was true for CAB5. Therefore, *BoGI* is related to low temperatures because the moment when the low temperature of *BoGI* increased, the expression was accelerated (Figure 4A). When comparing the CAB3 and CAB5 controls, CAB3 peaked one week earlier, and CAB5 rapidly peaked at one week as well in the low-temperature treatment group. This suggests the possibility that flowering time may also be different because the expression time of *BoGI* differs between species, even at the same light time or temperature. Notably, the expression of *COOLAIR*, which binds to *BoFLC* and inhibits its expression, increased when the expression of *BoFLC*

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decreased under cold treatment (Figure 4B). The expression of *COOLAIR* increased rapidly at 7 weeks when *BoFLC* homologous expression began to decrease in cold-treated cabbages. The expression level, which peaked at week 7, began to decrease sharply after that and remained constant. This indicates that *COOLAIR* is related to *BoFLC*.

We analyzed the structural characteristics of *FLC* homologs between *Brassica* varieties at different flowering times using allele-specific polymerase chain reaction (AS-PCR) amplification and cloning. Mutations present in each sequence occur because of a cis polymorphism in the *BoFLC* homolog, suggesting that this may be important for *FLC* transcription and may contribute to flowering time variations between early- and late-flowering varieties. In particular, it has been suggested that the previously known *BoFLC1*, as well as *BoFLC2* and *BoFLC3* genes, may have roles and functions related to flowering. In future studies, it will be helpful to develop cabbage varieties that control flowering through target genome editing techniques, such as CRISPR-CAS9 for the *BoFLC* allele identified in this study, as well as *Brassicaceae* varieties. In addition, markers for selecting varieties with different flowering times could be developed.

4. Materials and Methods

4.1. Plant materials and growth conditions

In this study, we used genetically fixed cabbage genotypes to obtain uniform nucleotide sequences. Three inbred lines or commercial cultivars such as *20FL-CAB1* (early-flowering with a time of 140–150 days*), 20FL-CAB3* (mid-flowering with time of 160–170 days), and *20FL-CAB5* (late-flowering with a time of ≥ 190 days) with distinct flowering times (varying by 40–45 days) were derived from a domestic seed company (Joeun Seeds Co., LTD, Goesan-gun, South Korea) in the Republic of Korea. Seeds of all the lines were sown and cultivated in a greenhouse (25 and 20 ºC under 16 and 8 h light and dark conditions, respectively) for 30 days in April 2020 and then transplanted to the field. Sixty days after field transplantation, the head portions of the cabbages were removed and transferred to plastic pots (30 × 25 cm) containing a mixture of a 1:1 ratio of cocopeat and soil. A set of pots without cold treatment (control) was allowed to grow under greenhouse conditions with 16 h of light at 25 °C and 8 h of dark at 18 °C. For the chilling or vernalization treatment, pots were transferred to incubators (TOGA clean system; model: TOGA UGSR01, Daejong, Korea) maintained at 4 °C with 10 h/14 h (light/dark) for 0–11 weeks. Leaf samples were collected from young leaves of plants grown under both the control and cold treatments each week (0–11 weeks of vernalization). The collected leaf samples were quickly frozen in liquid nitrogen and stored at − 80 °C for further use.

4.2. Genomic DNA extraction, PCR amplification, and sequencing of BoFLC homologs

Genomic DNA was extracted from young leaves using a WizPrep Plant DNA Mini Kit (WizBiosolutions, Seongnam, South Korea). According to the gene sequences of *BoFLC1* (AM231517.1), *BoFLC2* (AY306124.1), and *BoFLC3* (AY306125.1) deposited in GenBank, allele-specific (AS) PCR primers were designed and used to amplify *BoFLC*- encoding genes in cabbage. The primer pairs used in this study are listed in Table S2. PCR amplifications were conducted in a 50 μL reaction volume containing 100 ng DNA, 0.5 μM of both forward and reverse oligos, 0.5 μM of dNTP, 10x Ex *Taq* buffer, 0.8 units of *Ex Taq* polymerase (Takara) and autoclaved water. PCR reactions were performed on a thermal cycler using the following program: one cycle of 30 s at 98 °C; 30 cycles of 30 s at 98 °C, 30 s at 60 °C, 3 min 30 s at 72 °C; one cycle of 15 min at 72 °C. The PCR products were resolved on a 1% agarose gel in 1x TBE buffer, then stained with ethidium bromide, and visualized under a UV transilluminator. The expected amplicons were excised from the gel and purified using a LaboPass™ Gel Extraction Kit (Cosmo Genetech, South Korea) as recommended by the manufacturer. To sequence the purified amplicons, 2000 bp contig primers flanking the entire gene were designed for each *BoFLC* locus using the Primer3Plus online tool. PCR amplicons were sequenced using an ABI3730XL sequencer (Macrogen Co., Seoul, South Korea).

4.3. Sequence analyses and Phylogenetic analysis of BoFLC homologs

The *BoFLC1* genomic DNA sequences obtained in this study were deposited in the National Agricultural Biotechnology Information Center (NABIC; https://nabic.rda.go.kr/) database under the following numbers: NU-1420-000001 (*BoFLC1* from CAB1) and NU-1425-000001 (*BoFLC1* from CAB5). The sequencing results of nucleotide contigs were assembled, and comparative analysis among other *BoFLC* homologs to identify sequence variations (insertions and deletions) was conducted using BioEdit (version 7.2.5) sequence alignment software (Tom Hall, Ibis Biosciences, Carlsbad, CA, USA). The intron-exon organization of *BoFLC* homologs was predicted using the Gene Structure Display Server (GSDS2.0; http://gsds.cbi.pku.edu.cn/) by aligning the genomic sequences with their respective CDS sequences. Multiple sequence alignments of the deduced amino acid sequences of the identified *BoFLC* homologs were performed using CLUSTAL multiple sequence alignment in MUSCLE with the default parameters (http://www.ebi.ac.uk/Tools/msa/muscle/, version 3.8). To analyze the evolutionary relationships among *FLC* homologs from different *Brassica* species, a maximum likelihood tree and neighbor-joining tree (1000 replicates) were constructed using a dataset of *FLC* genes containing 31 deduced amino acid sequences, including those from cabbage in this study and those from other *Brassica* species (*B. rapa*, *B. nigra*, *B. oleracea* and *B. napus*) reported in previous studies[15,35]. The translated sequences of the whole coding regions of *FLC* were aligned using the MUSCLE method, and a tree was constructed using the neighbor-joining (NJ) method with MEGA version 10.0.5 and bootstrap analysis of 1000 replicates.

4.4. Extraction of mRNA and Expression profiling of Flowering genes

Total RNA from the leaf samples of both the control and treated plants was extracted using a GeneAll Hybrid RNA purification kit (GeneAll Biotechnology, Daejeon, Korea), as recommended by the manufacturer. cDNA was synthesized from 1.5 µg of RNA using the PrimeSrcript® RT reagent with a gDNA eraser kit (Takara Korea Biomedical, Seoul, Republic of Korea) according to the manufacturer's instructions. Primers specific for *BoFLC* and *Actin* genes were designed using the Primer3Plus online tool and were used for qRT-PCR analysis (Table 1). A total of 13 µL of reaction mixture containing 2 µL of cDNA template, 6 µL 2x SYBR green Q master LaboPassTM (COSMOgenetech, South Korea), and 0.5 µM of gene-specific forward and reverse oligonucleotide primers. To estimate the relative mRNA expression of levels of transcripts, qRT-PCR reactions were performed on a CFX96 Real-Time PCR Detection System (Bio-Rad) with the following parameters: one cycle for initial denaturation at 95 °C for 3 min, 40 cycles of 95 °C for 15 s, 60 °C for 20 s, 72 °C for 15 s, a cycle at 65 °C for 5 s, and a final cycle of 95 °C for 2 s to detect primer specificity based on melt curve analysis. The relative expression levels of target gene transcripts were estimated using the 2-ΔΔCt method [36] from three independent replicates.

4.5. Statistical Analysis

To analyze statistically significant differences in the relative expression of genes, ANOVA with Duncan's multiple range test was performed using SPSS version 21.0.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org and National Agricultural Biotechnology Information Center (NABIC; https://nabic.rda.go.kr/). Table S1: List of cabbage, kohlrabi, and broccoli materials used in this study with their flowering time. Table S2: List of primers used in this study. Table S3: Percent identity matrix of CDS multiple sequence alignment cabbage (FLC-CA1, -CA3, and -CA5), kohlrabi (FLC-KH7 and -KH8), and broccoli (FLC-BR10 and –BR11) (shown as in Table S1) having different flowering time. Figure S1: Comparisons of *BoFLC1* (A), *BoFLC2* (B), *and BoFLC3* (C) gene structure, respectively, between reference (AM231617.1) and early- (CAB1), Mid- (CAB3) and late- (CAB5) flowering lines.

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